

Isosilybin B causes androgen receptor degradation in human prostate carcinoma cells via PI3K-Akt-Mdm2-mediated pathway*

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Abstract:

The identification and development of novel nontoxic phytochemicals that target androgen and androgen receptor (AR) signaling remains a priority for prostate cancer (PCA) control. In the present study, we assessed the antiandrogenic efficacy of isosilybin B employing human PCA LNCaP (mutated AR), 22Rv1 (mutated AR) and LAPC4 (wild-type AR) cells. Isosilybin B (10–90 μ M) treatment decreased the AR and prostate specific antigen (PSA) levels in LNCaP, 22Rv1 and LAPC4 cells, but not in non-neoplastic human prostate epithelial PWR-1E cells. Isosilybin B treatment also inhibited synthetic androgen R1881-induced nuclear localization of AR, PSA expression and cell growth, and caused G1 arrest. In mechanistic studies identifying AR degradation, isosilybin B caused increased phosphorylation of Akt (Ser-473 and Thr-308) and Mdm2 (Ser-166), which was linked with AR degradation as pretreatment with PI3K inhibitor (LY294002)-restored AR level. Further, over-expression of kinase-dead Akt largely reversed isosilybin B mediated-AR degradation suggesting a critical role of Akt in AR degradation. Antibody pull-down results also indicated that isosilybin B treatment enhances the formation of complex between Akt, Mdm2 and AR, which promotes phosphorylation-dependent AR ubiquitination and its degradation by proteasome. Together, present findings identify a novel mechanism for isosilybin B-mediated anticancer effects in human PCA cells.

Keywords: androgen receptor; prostate specific antigen; prostate cancer; isosilybin B

Article:

Introduction

Androgen and androgen receptor (AR) mediated signaling plays a critical role in the development and progression of prostate cancer (PCA), which is the leading cause of cancer-related deaths in American men (Jemal et al., 2007). For last few decades, surgical or chemical ablation of androgens has been the frontline therapy for treating patients with locally advanced or metastatic PCA (Grossmann et al., 2001; Chen et al., 2004). Although most patients initially respond to these treatments, the disease eventually progresses to hormone-refractory state, which is caused mainly by AR overexpression, mutations or post-translational activation of the receptor or its coactivators (Grossmann et al., 2001; Chen et al., 2004; Sharifi and Farrar, 2006). These changes allow AR to become activated by low levels of adrenal androgens, other steroids and even antiandrogens or by ligand-independent mechanisms (Sharifi and Farrar, 2006). Therefore, aiming only at reducing the circulating levels of androgens or use of antiandrogens could be a major reason for an overall failure of endocrine therapy. Hence, ablation of AR from PCA cells using nontoxic phytochemicals could be an attractive therapeutic strategy for this deadly malignancy.

Silymarin is a crude extract of flavonolignans obtained from milk thistle (*Silybum marianum* (L.) Gaertn.) seeds, and is widely known for its hepato- protective and cancer chemopreventive action (Wellington and Jarvis, 2001; Singh and Agarwal, 2002, 2006; Deep et al., 2006). Recently, we have isolated and purified from silymarin seven distinct flavonolignans namely silybin A, silybin B, isosilybin A, isosilybin B, silydianin,

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silychristin, isosilychristin and one flavonoid taxifolin (Kim et al., 2003); and assessed their biological effects on many antiproliferative end points in human PCA cell lines (Davis-Searles et al., 2005). Isosilybin B ranked the most potent flavonolignan for nearly all end points including inhibition of prostate-specific antigen (PSA) secretion (Davis-Searles et al., 2005), which is used as a clinical tool in screening, diagnosis and treatment decisions for human PCA (Nelson et al., 2003). Here we conducted detailed mechanistic studies with isosilybin B focusing on AR by employing androgen-dependent LNCaP cells with single AR mutation (T877A), LAPC4 cells with wild-type AR and androgen-independent 22Rv1 cells with mutated AR (H874Y), though all the three cell lines have functional AR (Nagabhushan et al., 1996; Klein et al., 1997; Mendoza et al., 2002; Bernard et al., 2003).

Androgen receptor is a phosphoprotein and its activity is regulated through phosphorylation by many kinases. Various studies have shown the role of glycogen synthase kinase 30 (GSK30), stress kinase, mammalian target of rapamycin (mTOR) kinase and cyclin-dependent kinase 1 in regulating phosphorylation, stability and localization of AR (Wang et al., 2004; Cinar et al., 2005; Chen et al., 2006; Gioeli et al., 2006). Further, there are numerous studies suggesting the importance of Akt/PKB (a serine/threonine kinase) activation in AR degradation (Lin et al., 2001, 2002; Liao et al., 2005) as well as AR activation (Miyamoto et al., 2005; Reddy et al., 2006); though the interaction of Akt with AR largely remains unclear. Accordingly, we also examined the effect of isosilybin B on Akt phosphorylation and its possible role in AR degradation.

Results

Effect of isosilybin B and its other isomers on PSA and AR levels

Recently we showed the effect of isosilybin B and its other isomers namely silybin A, silybin B and isosilybin A (Figure 1 a) on secreted PSA levels in LNCaP cells after 72h treatment (Davis-Searles et al., 2005). Here, first we examined a comparative effect of these diastereoisomers, following 24h of their treatment, on both secreted and intracellular PSA levels in PCA LNCaP, 22Rv1 and LAPC4 cells. All four pure compounds (at 90 μ M) decreased PSA levels (secreted as well as intracellular) in LNCaP (Figure 1b), 22Rv1 (Figure 1c) and LAPC4 (Figure 1 d) cells, though isosilybin B was far more potent compared to silybin A, silybin B and isosilybin A (Figures 1b–d). The comparative superior efficacy of isosilybin B on secreted PSA inhibition in LNCaP, 22Rv1 and LAPC4 cells was also confirmed by ELISA assay (Figure 1e). Since AR (110 kDa in LNCaP cells and 114 kDa in 22Rv1 cells) is known to regulate the expression of androgen response element (ARE) containing genes including PSA (Miyamoto et al., 2005), next we examined a comparative effect of these compounds on AR levels. In all the three cell lines, isosilybin B strongly decreased the AR protein levels, while the effect of silybin A, silybin B and isosilybin A on AR levels was either marginal or was relatively lesser (Figures 1b–d). Together, these results suggest that among all the four isomers, isosilybin B is most effective in decreasing PSA and AR protein levels. Accordingly, we selectively focused our efforts to conduct detailed studies only with isosilybin B.

In LNCaP cells, isosilybin B (10–90 μ M) decreased AR and PSA (both secreted and cellular) levels after 24, 48 and 72h of treatment (Figure 2a). Similarly, isosilybin B treatment (60 and 90 μ M) decreased AR and PSA levels in 22Rv1 and LAPC4 cells after 24 and 48h of treatment (Figures 2b and c). Importantly, isosilybin B did not alter AR and cellular PSA levels in non-neoplastic human prostate epithelial PWR-1E cells under identical treatment conditions (Figure 2d), suggesting a selectivity in its response only toward human PCA cells. The secreted level of PSA was not detectable in PWR-1E cells (data not shown).

Isosilybin B decreases AR protein levels in both nuclear and cytoplasmic fractions

Since nuclear localization of AR is essential for its transcriptional activity (Miyamoto et al., 2005), we next evaluated the effect of isosilybin B on AR localization where its treatment for 6h slightly decreased the cytoplasmic and nuclear AR level (Figure 3a). Longer treatments (24–72h), however, strongly decreased both cytoplasmic and nuclear AR levels in a time-dependent manner (Figure 3a). We also observed a dose-dependent decrease in AR levels in both cytoplasmic and nuclear fractions after 72h of isosilybin B treatment (Figure 3b). A similar decrease in both cytoplasmic and nuclear AR levels was also observed in 22Rv1 cells following 24 and 48h of isosilybin B treatment (Figure 3c). Purity of nuclear and cytoplasmic fractions was confirmed by

reprobing the membranes with histone H1 and tubulin, detectable only in nuclear and cytoplasmic fractions, respectively.

PSA suppression by isosilybin B persists after its removal To assess whether the effect of isosilybin B on AR signaling is long lasting or reversible, LNCaP and 22Rv1 cells were treated with isosilybin B (90 μ M) for 24h and then conditioned medium was carefully removed, and cells were washed twice with fresh medium to remove both residual isosilybin B and secreted PSA. Fresh medium was then added and secreted PSA levels were measured as a function of time. As shown in Figure 3d, only 24h of isosilybin B treatment followed by wash-out study till 72 and 48 h in LNCaP and 22Rv1 cells, respectively, showed only a marginal reversal in the observed decrease in secreted PSA levels (Figure 3d). Similarly, cell number (LNCaP and 22Rv1) also remained significantly decreased even after isosilybin B withdrawal (Figure 3e).

Isosilybin B inhibits R1881-mediated nuclear localization/ expression of AR

Next we examined the effect of isosilybin B on synthetic androgen R1881 (methyltrienolone)-mediated nuclear localization/expression of AR. LNCaP cells grown in charcoal/dextran-treated fetal bovine serum (cFBS) were pretreated with 90 μ M isosilybin B for 2h and then stimulated with 1 nM R1881 for 150min and nuclear/cytoplasmic fractions were subsequently prepared. We observed an increased nuclear localization of AR with R1881, which was strongly reduced by isosilybin B (Figure 4a). We also validated these results employing confocal microscopy under identical treatment conditions. Here, fluorescein isothiocyanate (FITC) staining (for AR) was predominantly cytoplasmic in cells grown in cFBS, which becomes strongly nuclear in presence of R1881, and isosilybin B pretreatment followed by R1881 showed diffused FITC staining across both cytoplasm and nucleus (Figure 4a). In another experiment, 90 μ M isosilybin B was incubated with 1 nM R1881

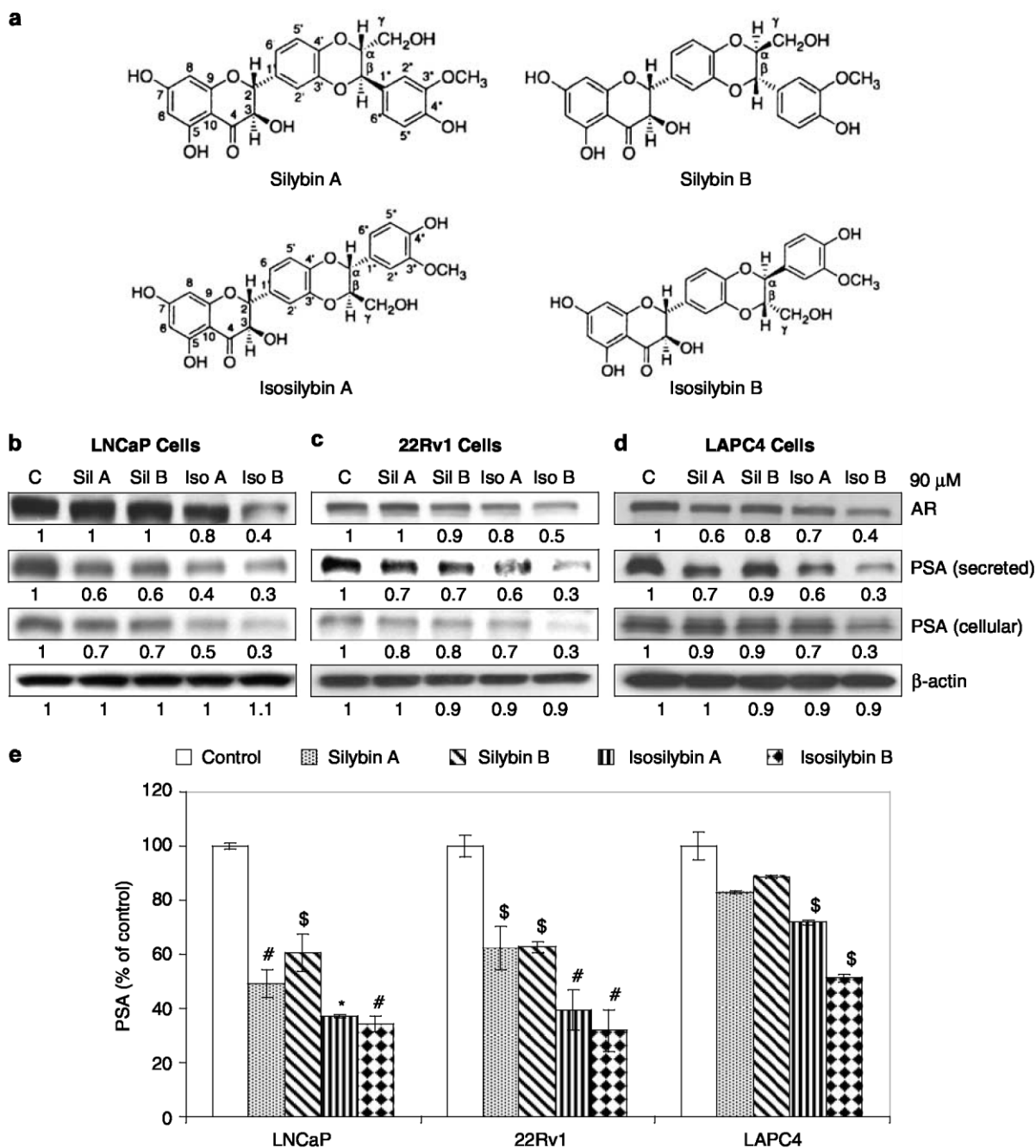


Figure 1 Silybin A, silybin B, isosilybin A and isosilybin B structures and their effect on androgen receptor (AR) and prostate specific antigen (PSA) levels in prostate cancer LNCaP, 22Rv1 and LAPC4 cells. (a) Chemical structure of silybin A, silybin B, isosilybin A and isosilybin B. (b–d) LNCaP, 22Rv1 and LAPC4 cells were treated with DMSO or 90 μ M silybin A (Sil A), silybin B (Sil B), isosilybin A (Iso A) or isosilybin B (Iso B) at 40–50% confluency. Cells were harvested 24 h later, lysates prepared and western blotting was carried out for AR and PSA (intracellular and secreted). Protein loading was monitored by stripping and reprobing the membranes with β -actin antibody. For secreted PSA, loading volume was normalized with respective protein values. The densitometry data presented below the bands are 'fold change' as compared with control after normalization with respective loading control (β -actin). Each experiment was repeated at least twice with similar results. (e) Media was also collected from control and treated samples (90 μ M dose of Sil A, Sil B, Iso A or Iso B) in the above mentioned experiment and PSA level was quantified using human PSA ELISA kit from R&D Systems. Each bar represents the value (mean \pm s.e.m.) from two experiments with each sample in duplicate. Each value was also adjusted with respective protein value. * $P \leq 0.001$; # $P \leq 0.01$; \$ $P \leq 0.05$.

in media for 10 min and then cells were treated with this mixture for 150 min, and both nuclear and cytoplasmic fractions were examined for AR levels. Under these conditions also, isosilybin B attenuated R1881-induced nuclear localization of AR (data not shown). In a related experiment, LNCaP cells were grown under 10% cFBS plus 1 nM R1881 for 4 days and isosilybin B was added for 24h. Here too, isosilybin B decreased R1881-mediated increase in AR level especially in nuclear fraction, as well as R1881-stimulated PSA

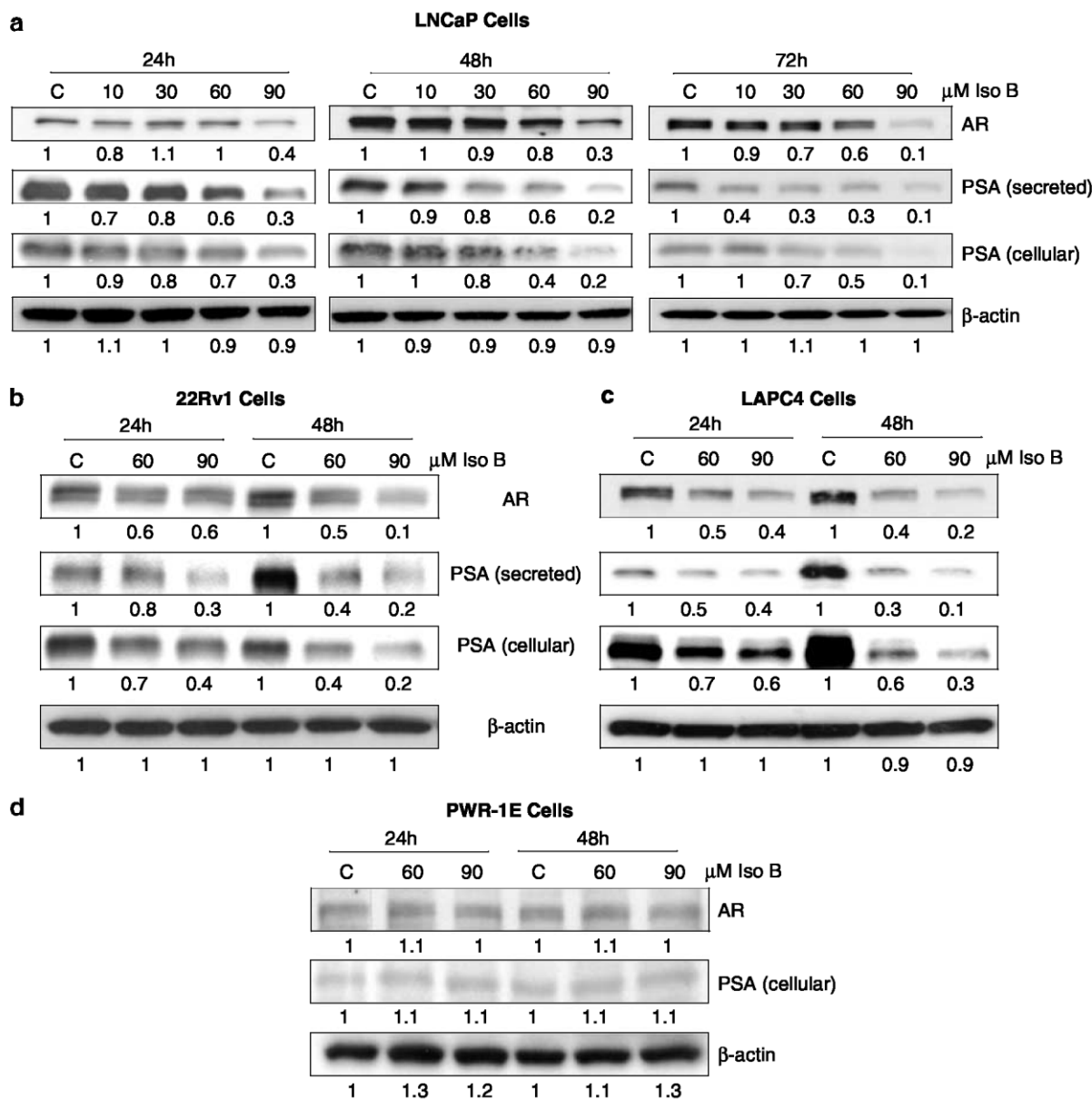


Figure 2 Effect of isosilybin B on androgen receptor (AR) and prostate specific antigen (PSA) levels in LNCaP, 22Rv1, LAPC4 and PWR-1E cells. (a-d) LNCaP, 22Rv1, LAPC4 and PWR-1E cells were treated at 40–50% confluency with DMSO or varying concentrations of isosilybin B (Iso B). At indicated treatment times, cells were harvested, lysates prepared and western blotting was carried out for AR and PSA (intracellular and secreted) as mentioned in experimental procedures. Protein loading was checked by stripping and reprobing the membranes with β -actin antibody. For secreted PSA, loading volume was normalized with respective protein values. The densitometry data presented below the bands are 'fold change' as compared with control after normalization with respective loading control (β -actin). Each experiment was repeated at least twice with similar results.

levels (Figure 4b) illustrating that isosilybin B inhibits androgen-induced AR signaling.

Isosilybin B inhibits R1881-stimulated growth of LNCaP cells

We also assessed the effect of isosilybin B treatment on androgen-stimulated growth of LNCaP cells, where compared to 10% FBS, cells grown in 10% cFBS showed a marked reduction in growth (Figure 4c). This was expected because cFBS is devoid of hormones and other growth promoting agents. Cells grown in 10% cFBS + 0.1 nM R1881 showed greater cell growth compared to cultures grown in cFBS (Figure 4c). Isosilybin B treatment (90μM) for 24h inhibited the majority of R1881-stimulated cell growth (Figure 4c).

Isosilybin B induces G1 arrest in serum- and androgen-stimulated LNCaP cells

The treatment of LNCaP cells grown in 10% FBS with isosilybin B resulted in significant G1 arrest (Figure 4d). Strong G1 arrest was also observed in cells grown in 10% cFBS. Next we examined whether the absence of

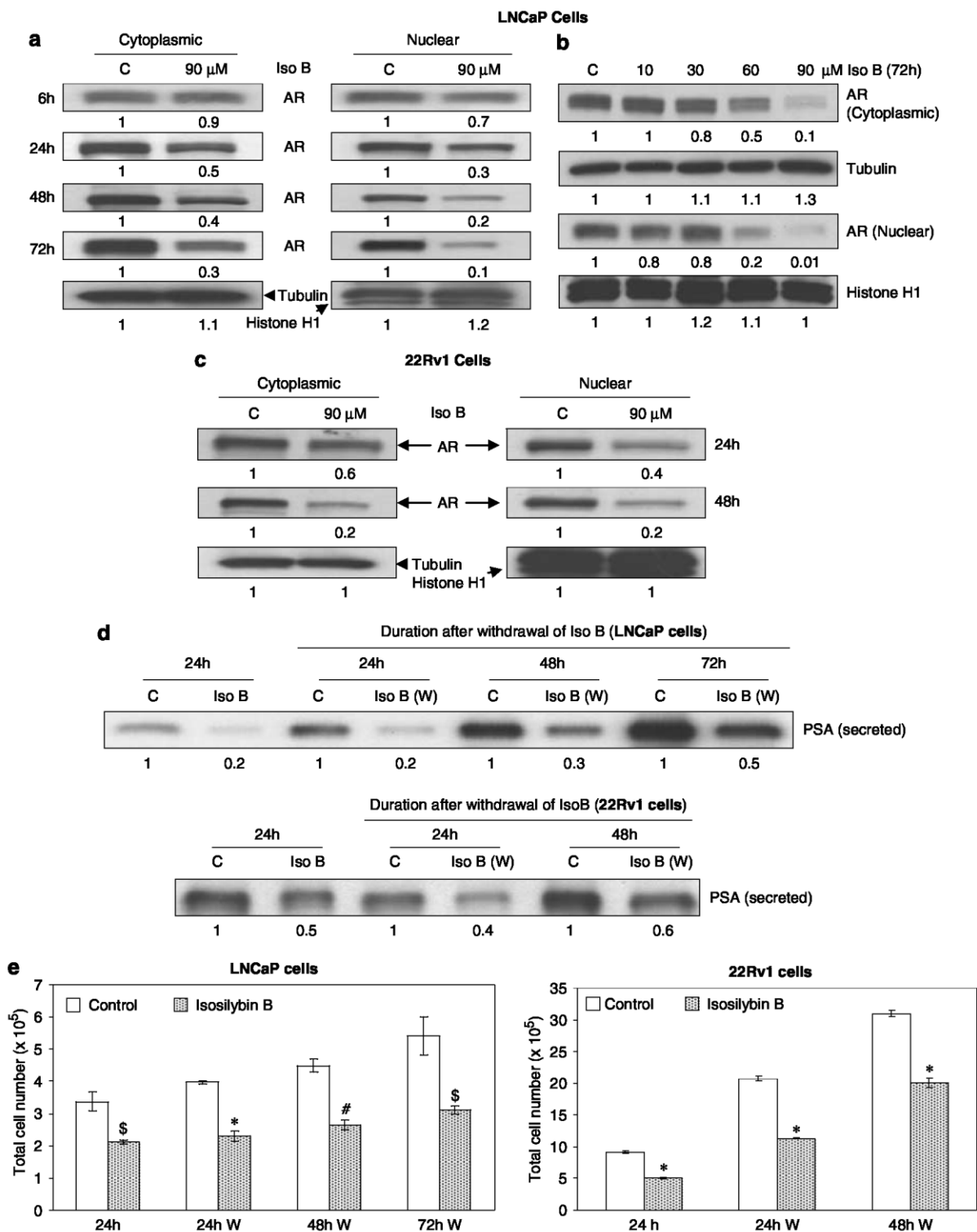


Figure 3 Effect of isosilybin B on nuclear/cytoplasmic levels of androgen receptor (AR) and sustained suppression of prostate specific antigen (PSA) secretion in LNCaP and 22Rv1 cells. (a and c) LNCaP or 22Rv1 cells were treated with DMSO or 90 μ M isosilybin B (Iso B) for indicated time (6–72 h). Cells were harvested, nuclear/cytoplasmic fractions were prepared and western blotting was carried out for AR as described in Materials and methods. (b) LNCaP cells were treated with various concentrations (10–90 μ M) of isosilybin B for 72 h. Cells were harvested and nuclear/cytoplasmic fractions were prepared and western blotting was carried out for AR. (d and e) LNCaP or 22Rv1 cells were treated with isosilybin B (90 μ M) for 24 h. Cells were washed twice with fresh medium to remove residual isosilybin B as well as PSA. Fresh media was added, and media was collected afterwards at regular intervals (24, 48 and 72 h) and analysed for PSA levels by western blotting. At each time point cell number was also counted as shown by bar diagram. Each bar is mean \pm s.e.m. of three samples for each treatment. For secreted PSA, loading volume was normalized with respective cell numbers. Each experiment was repeated at least twice with similar results. The densitometry data presented below the bands are 'fold change' as compared with control after normalization with respective loading control (β -actin or histone H1). W: Withdrawal of isosilybin B. * $P \leq 0.001$; # $P \leq 0.01$; \$ $P \leq 0.05$.

androgen in cFBS was a major factor for observed G1 arrest in 10% cFBS grown cells and that whether isosilybin B inhibits R1881-stimulated cell cycle progression. Compared with 10% cFBS alone, cells grown in 10% cFBS +0.1 nM R1881 showed a release from G1 arrest, but treatment of R1881-stimulated cells with isosilybin B restored cell cycle arrest (Figure 4d). These data suggest that growth inhibitory effects of isosilybin B could be due to its activity toward androgen-AR signaling.

Isosilybin B causes a decrease in AR half-life

We next focused our efforts on identifying whether post-translational modifications play a role in isosilybin B-mediated AR decrease. To address this issue, protein synthesis inhibitor cycloheximide (CHX) was added in LNCaP cells with or without isosilybin B (90 gM), and as a function of time cells were harvested and AR protein level measured by western blotting. In isosilybin B-treated LNCaP cells, the half-life of AR protein was reduced to 6.8 h from 13.8 h in control cells (Figure 5a), suggesting that the observed decrease in AR protein level by isosilybin B could be due to post-translational degradation. Based on this observation, next we focused our efforts on AR degradation by post-translational mechanisms.

Isosilybin B increases the phosphorylation of Akt

Akt (60 kDa) is one such kinase whose role has been described in the phosphorylation and degradation of AR (Lin et al., 2001, 2002). Therefore, we next examined the effect of isosilybin B treatment on Akt phosphorylation. Isosilybin B treatment of LNCaP cells increased phosphorylation levels of Akt at Ser-473 and Thr-308 sites (Figure 5b) without any changes in total Akt after 24h; however, at later time points (48 and 72h) and higher doses decreased the total Akt levels. So in further studies to understand the effect of isosilybin B treatment on Akt-AR interaction, we focused, on 24h or earlier time point. Similar to results in LNCaP cells isosilybin B treatment also caused a significant increase in Akt phosphorylation at Ser-473 site in 22Rv1 cells (Figure 5c). Together, these similar results in two different cell lines provided us further impetus to investigate the role of Akt in isosilybin B-mediated AR degradation.

Role of Akt in isosilybin B-mediated AR degradation To dissect the role of Akt activation in isosilybin B-mediated AR degradation, we pretreated LNCaP cells with 20 gM dose of PI3K inhibitor (LY294002), which resulted in inhibition of Akt phosphorylation (Figure 6a). When isosilybin B (90 gM) was added after PI3K inhibitor, a decrease in AR level was no longer evidenced (Figure 6a) and PI3K inhibitor also largely reversed isosilybin B-mediated decrease in PSA level (Figure 6a). Together, these results identified the crucial role of PI3K-Akt pathway in isosilybin B-mediated AR degradation. To further support this conclusion, we transfected LNCaP cells with 0.5 µg pCMV5-HA cAkt kinase mutant or pCMV5-HA (control) and treated with vehicle or isosilybin B. In HA-tagged control plasmid transfected cells, isosilybin B increased the Akt kinase activity and decreased the AR level (Figure 6b). However, in overexpressed kinase mutant Akt, we observed a comparatively much lesser decrease in AR level with isosilybin B, further suggesting an important role of Akt in isosilybin B-mediated AR decrease (Figure 6b). We also observed an increase in total Akt in the samples with overexpressed Akt kinase mutant compare to controls showing the efficiency of transfection (antibody used detects both wild-type and kinase mutant Akt; Figure 6b).

Isosilybin B increases AR phosphorylation

As we observed that isosilybin B causes Akt phosphorylation and increases its kinase activity as well, which is involved in AR degradation, and since Akt is known to phosphorylate and degrade AR (Lin et al., 2001, 2002), we next examined the effect of isosilybin B on phosphorylation status of AR at Ser-210/213 site in presence and absence of PI3K inhibitor. Indeed isosilybin B resulted in an increased phosphorylation of AR at Ser-210/213 site, which was inhibited in the presence of PI3K inhibitor (Figure 6a). We next examined whether Akt physically interacts with AR by immunoprecipitating Akt followed by western blotting for AR, as well as immunoprecipitating AR followed by western blotting with anti-pAkt Ser-473 antibody. In both the cases, we observed an increased binding between the two molecules (Figure 6c). We also immunoblotted Akt and AR immunoprecipitates with their respective antibodies to support the validity of pull-down Akt and AR (data not shown). In addition, the specificity of the binding was confirmed by immunoprecipitation with normal immunoglobulin G (IgG), where no binding was observed (data not shown). These results suggest that

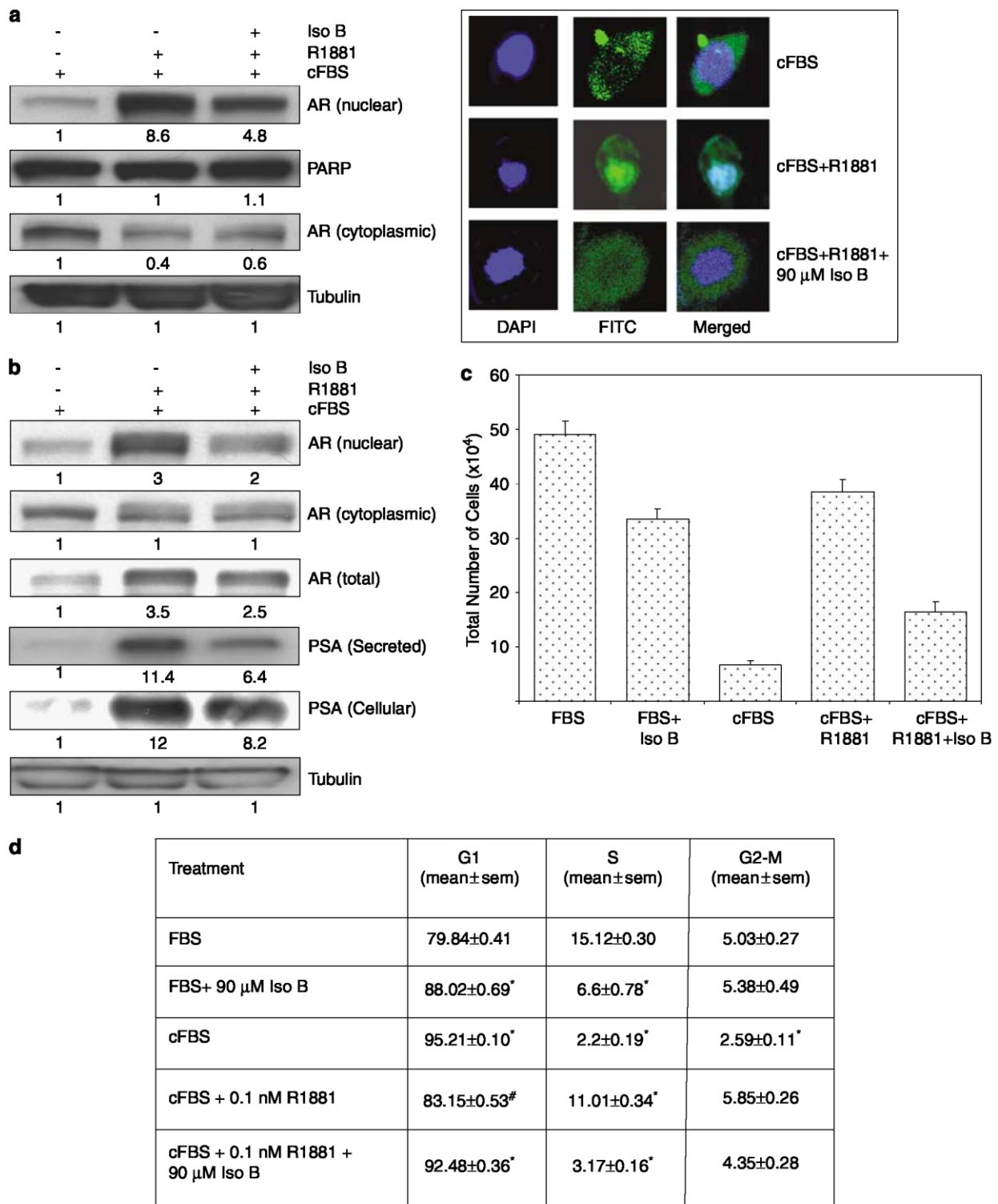
isosilybin B treatment increases the binding between these two molecules that might result in AR phosphorylation.

Isosilybin B increases Mdm2 phosphorylation and interaction with both Akt and AR

Mdm2 (90 kDa) is a ring-finger protein whose E3-ligase activity plays a crucial role in ubiquitination and degradation of a number of proteins including AR (Zhou et al., 2001; Lin et al., 2002; Chen et al., 2005).

Accordingly, we next examined the effect of isosilybin B on Mdm2 phosphorylation at Ser-166 site, which is associated with its activation and enhancement of ubiquitin ligase activity (Zhou et al., 2001; Lin et al., 2002; Paajarvi et al., 2005). Isosilybin B increased Mdm2 phosphorylation level at Ser-166 site in both LNCaP and 22Rv1 cells (Figures 5b and c) without any change in total Mdm2 levels in 22Rv1 cells but in LNCaP cells we observed an increase in total Mdm2 levels after 48 and 72 h of treatment (Figures 5b and c).

Earlier studies have also shown that Akt physically interacts with Mdm2 and phosphorylates it at Ser-166 site (Zhou et al., 2001; Lin et al., 2002). Therefore, we next examined whether isosilybin B-mediated Akt activation had any role in Mdm2 phosphorylation. The downregulation of Akt activation using LY294002 also resulted in an inhibition of isosilybin B-mediated phosphorylation of Mdm2 at Ser-166 (Figure 6a). Further, isosilybin B treatment significantly increased the binding between Mdm2 and Akt (Figure 6c). Similarly, we also observed an increased binding between Mdm2 and AR following isosilybin B treatment (Figure 6c). We also immunoblotted Mdm2 immunoprecipitates with its antibody to support the validity of pull-down Mdm2 (data not shown). In addition, the specificity of the binding was confirmed by immunoprecipitation with normal IgG, where no binding was observed (data not shown). Together, these results suggested that isosilybin B-mediated Akt



activation leads to Mdm2 activation and its increased interaction with AR, which might lead to ubiquitination of AR followed by its degradation.

Isosilybin B-triggered AR degradation is proteasome-mediated

Protein ubiquitination provides the recognition signal for 26S proteasome leading to protein degradation (Hershko and Ciechanover, 1998). We next examined the role of ubiquitination and proteasomal activity in isosilybin B-mediated AR degradation. LNCaP cells were treated with 90 gM isosilybin B for 20 h and then 20 gM 26S proteasomal inhibitor (MG132) was added. Cell lysates were prepared after 4 h of MG132 treatment and AR level was determined through western blotting. MG132 treatment largely restored the AR level decreased by isosilybin B (Figure 6d). Since inhibition of proteasomal activity by MG132 leads to an increase

in polyubiquitinated form of AR, we stripped and reprobed the same blot with anti-ubiquitin antibody. There was an increase in the extent of ubiquitinated protein in the lanes treated with MG132 with or without isosilybin B treatment (Figure 6d). This result indirectly shows that MG132 treatment resulted in accumulation of ubiquitinated form of AR as proteasomal activity is inhibited; suggesting that isosilybin B-mediated AR degradation is largely proteasome dependent.

Discussion

Hormone-refractory relapse is an inevitable and lethal event for advanced PCA patients after hormone deprivation (Lee and Chang, 2003a; Chen et al., 2004). Notably, AR not only mediates the effect of androgen on tumor initiation but also plays major role in the relapse transition (Grossmann et al., 2001; Lee and Chang, 2003a; Chen et al., 2004). This provides a strong rationale for searching new effective agents targeting the downregulation of AR to treat or prevent advanced PCA progression. Here, we have shown that isosilybin B treatment strongly decreases AR levels in human PCA LNCaP, 22Rv1 and LAPC4 cells.

Androgen receptor is a nuclear receptor which binds to ARE and turns on its target genes regulating diverse cell functions such as cell growth and apoptosis (Grossmann et al., 2001). Isosilybin B treatment strongly inhibited the translocation of AR to the nucleus, which is, in turn, evident by a strong decrease in the expression levels of PSA. The present study also suggests that isosilybin B possesses strong antiandrogenic action as it inhibited R1881-induced AR expression/nuclear translocation, PSA expression and cell growth. The *in vitro* pre-incubation of isosilybin B with R1881 also inhibited nuclear translocation of AR, which raises the possibility that isosilybin B can bind and sequester R1881. Alternatively, isosilybin B might also compete with R1881 for its binding with AR; at present it is not clear whether isosilybin B can interact directly with either wild-type or mutated AR. However, precedent exists for flavonolignan actions on other steroid hormone receptors. Silybin B exhibited selective partial agonist activity on estrogen-receptor-mediated reporter gene expression (EC₂₅ = 4.4 gM, 30% E₂max induction) and the silymarin mixture exhibits ERR binding activity (Seidlova-Wuttke et al., 2003; Pliskova et al., 2005). Another possibility is that isosilybin B may indirectly influence AR by targeting hsp90 chaperone, hsp90 cochaperones and/or other coregulators of AR, which play important role in proper folding, stability and nuclear translocation of AR (Lee and Chang, 2003b; Reddy et al., 2006). Indeed, these mechanisms and associated biological events require further investigation.

Earlier studies have shown that downregulation of AR, using siRNA, antisense oligonucleotides or ribozymes, leads to an inhibition of PCA cell growth (Tong et al., 2003; Haag et al., 2005). In the present study, we observed a strong G1 arrest and growth inhibition by isosilybin B in the presence of serum or androgen in LNCaP cells. Consistent with these biological effects, recently we have reported that isosilybin B strongly inhibits cell growth by modulating cell cycle regulators and by activating caspases in both LNCaP and 22Rv1 cells (Deep et al., 2007). There is a strong possibility that these growth inhibitory effects of isosilybin B might be due to antagonism of the androgen-AR signaling in both LNCaP and 22Rv1 cells.

As mentioned earlier, AR activation is regulated by phosphorylation at multiple sites, which is regulated by many kinases. In the present study, we identified the role of Akt in isosilybin B-mediated AR degradation. It has been shown that increased phosphorylation of Akt is

Figure 4 Isosilybin B inhibits R1881-induced androgen receptor (AR) nuclear localization, prostate specific antigen (PSA) expression and cell growth. (a) LNCaP cells grown in cFBS were pretreated with isosilybin B (90 μ M) for 120 min and subsequently R1881 was added for 150 min. Nuclear and cytoplasmic extracts were prepared and western blotting was performed for AR. Protein loading was monitored by stripping and reprobing the membranes with antibodies to tubulin and PARP. Each experiment was repeated at least twice with similar results. In another study, LNCaP cells were grown in 4-chambered slides and treated similarly as above, and then processed as detailed in 'Materials and methods' and cell images were captured using an inverted Nikon Confocal microscope. (b) LNCaP cells were grown in cFBS with or without synthetic androgen R1881 (1 nM) for 4 days and were treated with isosilybin B (90 μ M) for the next 24 h. Whole cell lysate or nuclear/cytoplasmic extracts were prepared and western blotting was performed for AR and PSA. For measuring secreted PSA, loading volume was normalized with respective protein value or cell number. The densitometry data presented below the bands are 'fold change' as compared with control after normalization with respective loading control (PARP or tubulin). (c and d) LNCaP cells were grown in cFBS with or without synthetic androgen R1881 (1 nM) for 4 days and were treated with isosilybin B (90 μ M) for next 24 h. Cells were then collected by brief trypsinization and analysed for total cell number and cell cycle phase distribution as detailed in Materials and methods. The data shown are mean \pm s.e.m. of three samples for each treatment. These results were similar in two independent experiments. * $P \leq 0.001$; # $P \leq 0.01$.

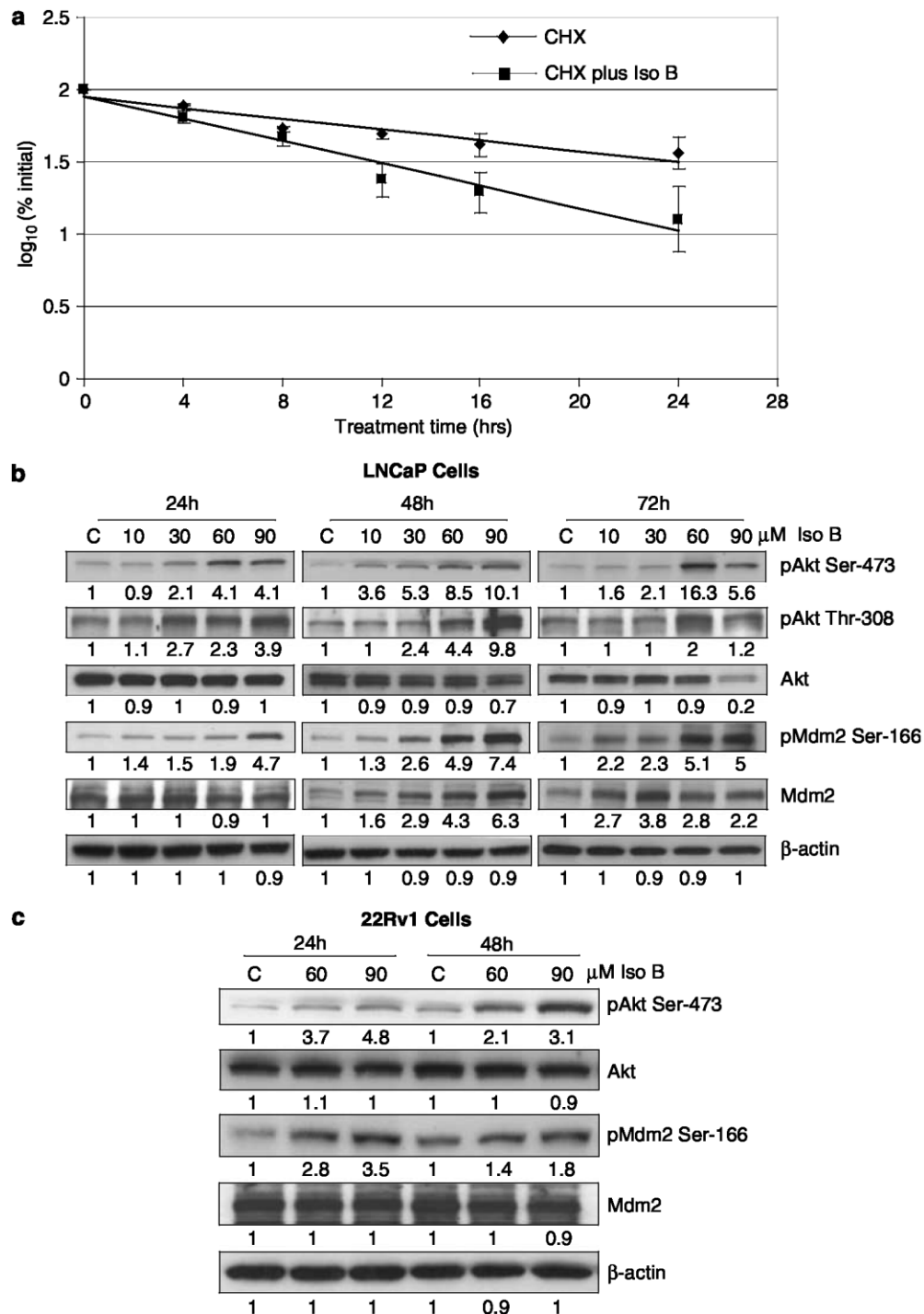


Figure 5 Isosilybin B regulates androgen receptor (AR) levels at post-translational level via phosphorylation of Akt and Mdm2. (a) LNCaP cells were grown to 60–70% confluency, and cycloheximide ($50 \mu\text{g ml}^{-1}$) was added with or without $90 \mu\text{M}$ isosilybin B at 0 h. Cells were harvested at various time points (0, 4, 8, 12, 16, 24 h), cell lysates prepared and AR protein level was determined by western blot analysis. Each membrane was stripped and reprobed with anti-tubulin antibody to normalize for differences in protein loading. Bands were scanned with Adobe Photoshop 6.0 (Adobe Systems Inc., San Jose, CA, USA), and mean density of each band was analysed by Scion Image program (NIH, Bethesda, MD, USA) and normalized with respect to tubulin (loading control). Each value in the scatter diagram is the average value obtained from three independent experiments. (b and c) LNCaP and 22Rv1 cells were treated with DMSO or varying concentrations of isosilybin B for indicated time. Cells were then harvested, lysates prepared and western blotting carried out for pAkt Ser-473, pAkt Thr-308, Akt, pMdm2 Ser-166 and total Mdm2. Protein loading was checked by stripping and reprobing the membranes with β -actin antibody. The densitometry data presented below the bands are 'fold change' as compared with control after normalization with respective loading control (β -actin). Each experiment was repeated at least twice with similar results.

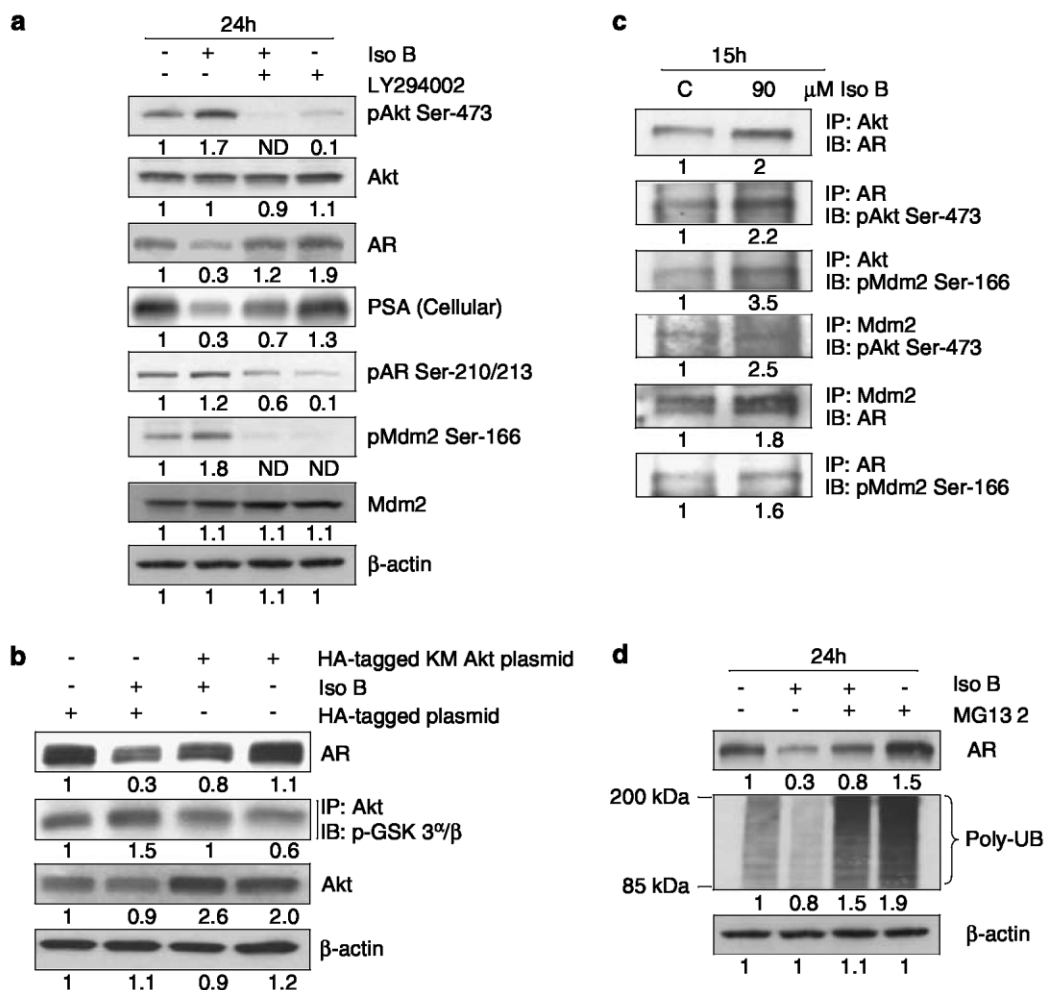


Figure 6 Isosilybin B-mediated androgen receptor (AR) decrease involves Akt, Mdm2 and ubiquitin-proteasome system. (a) LNCaP cells were pretreated with LY294002 (20 μ M) for 2 h, then treated with isosilybin B for 24 h, and cell lysates were prepared. Western blotting was carried out for pAkt Ser-473, Akt, AR, prostate specific antigen (PSA), pAR Ser-210/213, pMdm2 Ser-166 and Mdm2. (b) LNCaP cells were transfected with 0.5 μ g pCMV5-HA cAKT Kinase Mutant or pCMV5-HA (control) plasmids (gift of Dr AD Cox) at about 40–50% confluency and were treated with 90 μ M isosilybin B after 24 h of transfection. Cell lysates were prepared and analysed for AR level and kinase activity of Akt as detailed in Materials and methods. (c) LNCaP cells were treated with 90 μ M isosilybin B and cell lysates prepared after 15 h of treatment. For binding assays AR, Akt or Mdm2 were immunoprecipitated with appropriate primary antibodies followed by immunoblotting with anti-AR, pAkt Ser-473, Akt, pMdm2 Ser-166 or Mdm2 antibody as mentioned in experimental procedures. (d) LNCaP cells were treated with isosilybin B for 20 h and then with 20 μ M MG132 for 4 h. Cell lysates were prepared and analysed for AR and ubiquitination by western blotting as mentioned in methods. The relative mobility of molecular weight standards corresponding to 85 and 200 kDa is shown. Protein loading was checked by stripping and reprobing the membranes with β -actin antibody. The densitometry data presented below the bands are 'fold change' as compared with control after normalization with respective loading control (β -actin). ND: Not detectable. Each experiment was repeated at least twice with similar results.

associated with loss of AR during the N-methylN-nitrosourea induced prostate carcinogenesis (Liao et al., 2005). Sequence analysis of AR reveals two Akt consensus sequences (RXRXXS/T; Wen et al., 2000), thus Akt can interact directly with AR leading to its phosphorylation (Wen et al., 2000; Lin et al., 2001, 2002). We also observed that isosilybin B treatment results in the activation of Akt as evident by its increased phosphorylation and an increase in Akt kinase activity (Figure 6b) along with an increased binding between Akt and AR (Figure 6c). These molecular alterations possibly were involved in an increased phosphorylation of AR together with its degradation.

Other than acting directly, Akt can also influence AR level through other mechanisms; for example, Akt activation can activate mTOR, which has been reported to suppress AR level via post-transcriptional modifications (Cinar et al., 2005). Further, Akt activation can also decrease AR expression by reducing the levels of transcriptional factors, such as FOXO3a (Yang et al., 2005). Indeed, all these possibilities need to be examined in more details in future studies to further define the precise mechanisms underlying isosilybin B-mediated AR decrease via Akt activation.

In the present study, we also observed that isosilybin B increases Akt-mediated phosphorylation of Mdm2.

It has been shown earlier that AR ubiquitination is significantly impaired in Mdm2-deficient mouse embryonic fibroblasts (MEFs) compared with Mdm2- intact MEFs (Lin et al., 2002). Further, it seems that Mdm2 uses its RING-finger domain to interact with AR, and our binding assays clearly show that isosilybin B increases the interaction between AR and Mdm2 (Figure 6c), which might lead to ubiquitination of AR and its proteasomal-mediated degradation (Figure 6d). Earlier studies have shown that in response to DNA damage, Mdm2 levels are increased in cells with wild- type p53 (Pan and Chen, 2003). Furthermore, the roles of MAP kinase and mTOR have also been suggested in the induction of Mdm2 levels (Paajarvi et al., 2005). Accordingly, more studies are needed to investigate these pathways, which might contribute to isosilybin B-mediated increased levels of Mdm2 phosphorylation and their relevance in the observed AR decrease by isosilybin B.

In conclusion, the present study shows that isosilybin B strongly decreases AR levels in both androgen-dependent (LNCaP and LAPC4) and androgen-independent (22Rv1) PCA cells. Further, the present work for the first time suggested the role of activated Akt in AR degradation by a phytochemical, therefore, needs greater attention. Overall, the results from the present work provide a strong rationale for future investigation evaluating the efficacy of isosilybin B against PCA and associated mechanisms.

Materials and methods

Reagents

Silybin A, silybin B, isosilybin A, isosilybin B were isolated (purity between 98 and 100%) from silymarin (Madaus AG, Cologne, Germany) as described (Kim et al., 2003). LNCaP, 22Rv1 and PWR-1E cells were from ATCC (Manassas, VA, USA). LAPC4 cells were kindly provided by Dr RE Reiter (UCLA, CA, USA). All cell culture materials were from Invitrogen (Gaithersburg, MD, USA); cFBS was from Hyclone (Logan, UT, USA). Antibodies for AR, Akt, Mdm2, normal mouse and rabbit IgG, and protein A/G agarose beads were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Akt kinase assay kit (nonradioactive) and antibodies for pAkt (Ser-473, Thr-308), Akt, pMdm2 Ser-166 and anti-rabbit peroxidase conjugated secondary antibody were from Cell Signaling (Beverly, MA, USA). PSA antibody was from Dako Corporation (Carpinteria, CA, USA). Antibody for pAR Ser-210/213 was from Imgenex (San Diego, CA, USA). Cycloheximide, propidium iodide, 4',6-diamidino2-phenylindole (DAPI), and antibody for G-actin were from Sigma (St Louis, MO, USA). R1881 was from Perkin-Elmer (Wellesley, MA, USA). MG132 and LY294002 were from Biomol (Plymouth, PA, USA). Expression plasmids with dominant negative, kinase mutant Akt cDNA (pCM V5-HA-cAKT- KM) and pCMV5-HA (control) were kindly provided by Dr AD Cox, University of North Carolina at Chapel Hill. GeneJuice transfection reagent was from Novagen (Madison, WI, USA). Human PSA ELISA kit was from R&D Systems. ECL detection system and anti-mouse peroxidase conjugated secondary antibody were from GE Healthcare (Buckinghamshire, UK).

Cell culture and treatments

LNCaP, 22Rv1 and PWR-1E cells were cultured as described earlier (Deep et al., 2007) and LAPC4 cells were cultured in Iscoves's modified Dulbecco's medium with 10% FBS, and 1 % penicillin-streptomycin (P-S). Cells were treated with different concentrations (10–90 gM) of isosilybin B, and/or isosilybin A, silybin A and silybin B (90 gM) in dimethyl sulfoxide (DMSO) for desired time. An equal amount of DMSO (vehicle) was present in each treatment including control, which did not exceed 0.1 % (v/v). LNCaP cells were also cultured in phenol red-free medium containing 10% cFBS and 1% P-S with or without synthetic androgen R1881 for 4 days, and isosilybin B was then added for further 24h. Media samples were separately collected, and cells were processed for whole-cell lysate, cytoplasmic and nuclear extracts, FACS analysis, or for cell viability assay as mentioned earlier (Muller et al., 1989; Zi et al., 1998; Zi and Agarwal, 1999).

Western immunoblotting, and protein –protein binding and kinase assays

For western blotting, 40–70 gg of protein per sample was denatured in 2 x SDS –polyacrylamide gel electrophoresis (PAGE) sample buffers and subjected to SDS–PAGE on 6, 8 or 12% polyacrylamide tris-

glycine gels followed by immunoblotting as described earlier (Muller et al., 1989; Zi et al., 1998). Protein–protein binding studies were conducted as described earlier (Zi et al., 1998). Kinase activity assay for Akt was conducted as per vendor's protocol with some modifications (Dhanalakshmi et al., 2005).

Immunofluorescence staining and confocal imaging

LNCaP cells were grown in 4-chamber slides in phenol red-free medium with 10% cFBS, and were pretreated with isosilybin B (90 gM) or DMSO for 120 min followed by synthetic androgen R1881 (1 nM) for another 150 min. Cells were then fixed in 2% paraformaldehyde overnight at 4°C, permeabilized with 0.1 % Triton X-100 for 15 min and then incubated with goat serum. Cells were washed with PBS containing 0.25% BSA and incubated with AR antibody for 1 h followed by 45 min incubation with fluorescein-tagged secondary antibody, and counterstained with DAPI for 5 min. Cell images were captured at x 400 magnification on a Nikon inverted confocal microscope using 488/402 nm laser wavelengths to detect fluorescein and DAPI emissions, respectively.

Transfection

LNCaP cells were transfected using GeneJuice as per vendor's protocol. Briefly, cells were plated in 60 mm dishes and were transfected at about 50–60% confluency where 0.5 gg of pCMV5-HA cAkt KM (kinase mutant) or pCMV5-HA (control) was incubated with GeneJuice/serum free medium mixture for 15 min and then added dropwise to the cells. Cells were treated with DMSO or isosilybin B after 24h of transfection, and cell lysates prepared after 24h.

ELISA assay for PSA

ELISA assay for quantitative estimation of PSA in the media from LNCaP, 22Rv1 and LAPC4 cells was performed as per vendor's protocol (R&D Systems, Minneapolis, MN, USA).

Statistics

Statistical analysis was performed using SigmaStat 2.03 software (Jandel Scientific, San Rafael, CA, USA). Data were analysed using Student's t-test, and a statistically significant difference was considered to be at $P < 0.05$. For all results autoradiograms/bands were scanned with Adobe Photoshop 6.0 (Adobe Systems Inc., San Jose, CA, USA) and the mean density of each band was analysed by the Scion Image program (National Institutes of Health (NIH), Bethesda, MD, USA). In each case, blots were subjected to multiple exposures on the film to ensure that the band density was in linear range.

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